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A Chemical Genetic Screen in Zebrafish for Pathways Interacting with *cdx4* in Primitive Hematopoiesis

Elizabeth J. Paik,* Jill L.O. de Jong,* Emily Pugach, Praise Opara, and Leonard I. Zon

Abstract

cdx4, a caudal-related homeodomain-containing transcription factor, functions as a regulator of *hox* genes, thereby playing a critical role in anterior–posterior (A–P) patterning during embryogenesis. In zebrafish, homozygous deletion of the *cdx4* gene results in a mutant phenotype known as *kugelig*, with aberrant A–P patterning and severe anemia characterized by decreased *gata1* expression in the posterior lateral mesoderm. To identify pathways that interact with *cdx4* during primitive hematopoiesis, we conducted a chemical genetic screen in the *cdx4* mutant background for compounds that increase *gata1* expression in *cdx4* mutants. Among 2640 compounds that were tested, we discovered two compounds that rescued *gata1* expression in the *cdx4*-mutant embryos. The strongest rescue was observed with bergapten, a psoralen compound found in bergamont oil. Another member of the psoralen family, 8-methoxypsoralen, was also found to rescue *gata1* expression in *cdx4*-mutant embryos. The psoralen compounds also disrupted normal A–P patterning of embryos. These compounds modify the *cdx4*-mutant phenotype and will help elucidate signaling pathways that act downstream or parallel to the *cdx4*-*hox* pathway.

Introduction

BECAUSE OF THEIR SMALL SIZE, optical clarity, and substantial fecundity, zebrafish embryos are excellent tools for testing the effects of chemicals *in vivo* in a vertebrate animal model. Several recent studies have proven the efficacy of chemical screening using zebrafish embryos, identifying small molecules regulating hematopoietic stem cell emergence as well as other developmental pathways.^{1–11} Because most developmental and signaling pathways are conserved between mammals and teleosts, chemicals discovered using this approach in zebrafish are likely to be applicable to humans. In addition, characterizing the mechanism of action of compounds discovered in this type of screen may lead to new insights into the function of a specific gene of interest.

To date, most of the published chemical screens using zebrafish have been performed using wild-type embryos. Another advantage of the zebrafish model is the availability of many mutant strains and transgenic strains with phenotypes analogous to human diseases, which can be utilized in chemical screens.¹² Two such chemical modifier screens have been published to date. The first took advantage of a vascular

mutant called *gridlock*, with a mutation in the *hey2* gene.¹ The second utilized the *crash-and-burn* mutant, with a defect in cell cycle regulation.² Performing a chemical screen on a mutant background allows selection of compounds that rescue the phenotype of interest, with the hope of identifying modifying signaling pathways and potentially finding therapeutically relevant molecules.

The zebrafish has also proven to be an excellent model to study blood development, and forward genetic screens have generated numerous blood mutants that have advanced the understanding of developmental hematopoiesis.^{13,14} As in mammals, hematopoiesis in the zebrafish consists of two waves. The primitive or embryonic wave occurs during the first 24 h postfertilization, specifying blood cell development in the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM). Blood cells born in the ALM region become myeloid cells, expressing the myeloid transcription factor *pu.1*, whereas the PLM region gives rise to some myeloid cells but mostly erythroid cells, a process that is governed by the transcription factor *gata1*.^{15–17} Definitive hematopoiesis starts later and generates hematopoietic stem cells, which will produce all blood cell types for the animal's lifetime.

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The zebrafish mutant *kugelig*, with a deletion in the caudal-related homeobox gene *cdx4*, has aberrant anterior–posterior (A-P) patterning and *hox* gene expression, in addition to severe anemia due to failed specification of PLM cells into *gata1*+ erythroid cells.¹⁸ At the 10-somite stage, *cdx4* mutants show decreased expression of *gata1* in the PLM. This defect in primitive hematopoiesis can be partially rescued by 4-diethylamino benzaldehyde (DEAB), an inhibitor of retinoic acid (RA) biosynthesis¹⁹ (J.L.O. de Jong and L.I. Zon, unpublished data).

In this study, we conducted a chemical screen using *cdx4*-mutant embryos to seek new pathways that interact with *cdx4* during primitive hematopoiesis. A total of 2640 compounds were tested, revealing two that rescued *gata1* expression in *cdx4*-mutant embryos. Bergapten, also known as 5-methoxypsoralen (5-MOP), produced the most robust rescue of all the compounds tested. Bergapten was found to have effects similar to DEAB, including changes in A-P patterning. Neither bergapten nor DEAB significantly alters the expression of other blood genes including *flil1*, *scl*, and *pu.1* in wild-type embryos. However, similar to the rescue of *gata1* expression, bergapten and DEAB both rescue the expression of *pu.1* in *cdx4*-mutant embryos. The related compound 8-methoxypsoralen (8-MOP) was also shown to affect A-P patterning and to rescue loss of *gata1* and *pu.1* in *cdx4*-mutant embryos. This indicates that psoralen compounds may interact with *cdx4*-*hox* downstream pathway or a parallel pathway.

Methods

Zebrafish care and mutant lines

Wild-type AB strain, and *kugelig* heterozygote zebrafish carrying the *cdx4*-mutant allele were bred and maintained using standard zebrafish husbandry.²⁰ Developmental staging was done by embryo morphology.²¹ All zebrafish experiments and procedures were performed as approved by the Children's Hospital Boston Institutional Animal Care and Use Committee.

Chemical library

Compounds from the known bioactives collection (NINDS Custom Collection 2, Biomol 3, Prestwick 1, and Biomol 4) were obtained from the Institute of Chemistry and Cell Biology (ICCB) Longwood screening facility at Harvard Medical School. A total of 2640 compounds were screened. The ICCB compounds were stored at -20°C , dissolved in 100% dimethyl sulfoxide (DMSO) in desiccated storage containers in 384-well plates. All chemical transfers were performed robotically. One microliter of each compound dissolved in 100% DMSO was diluted into 300 μL of embryo medium (E3 fish-water)²⁰ with 1% penicillin/streptomycin (Invitrogen) and 1% DMSO in a 48-well nontissue culture-treated plate (Falcon). In addition, each 48-well plate contained blank wells used as negative control wells (E3 water with 1% DMSO and 1% Pen/Strep) and positive control wells with 20 μM DEAB.

Chemicals

Stock solutions were prepared as follows: DEAB (Sigma-Aldrich) was dissolved at 1 M concentration in DMSO (Sigma-Aldrich). Bergapten (Sigma-Aldrich) was dissolved in acetone at 5 mM concentration. 8-MOP (Sigma-Aldrich)

was dissolved in acetone at 5 mM concentration. 5-(4-Phenoxybutoxy)psoralen (PAP-1) (Sigma-Aldrich) was dissolved in DMSO at 7.25 mM concentration. All stock solutions were stored at -20°C , and fresh dilutions of working concentrations were prepared on the day of experiments.

Screening procedure

cdx4 heterozygous fish were mated every 1–2 weeks to generate embryos for screening. Males and females were set up overnight and kept separated until morning when they were allowed to mate and the embryos were collected. Embryos were kept at 28°C until treatment with chemicals. When embryos reached the dome stage, dead and unfertilized embryos were discarded, and healthy stage-synchronized embryos were pooled. At the 50% epiboly stage, approximately 20 embryos per well were arrayed in the 48-well plates containing one compound per well. As *cdx4* affects early mesodermal patterning, embryos were treated from the beginning of gastrulation (50% epiboly stage) until the 10-somite stage to find compounds that could overcome the need for *cdx4* in specifying primitive blood cells. Following the incubation at 21°C for approximately 14–15 h, embryos were dechorionated by incubation in pronase 167 $\mu\text{g}/\text{mL}$ (Roche) for 6 min. They were then washed repeatedly with E3 water to remove the chorions and the treatment chemicals and then fixed in 4% paraformaldehyde for whole-mount *in situ* hybridization with *gata1* and *cdx4* riboprobes (Fig. 1). The bilateral mRNA expression of *gata1*, a transcription factor critical for erythroid development, was used as the readout for the screen, with positive hits showing increased *gata1* in *cdx4*-mutant embryos. *cdx4*-mutant embryos were easily identified in each well by the lack of *cdx4* RNA expression in the midline and the tail bud (Fig. 1).

After *in situ* hybridization, embryos were transferred into 24-well plates to minimize optical distortion from the smaller wells in the 48-well plates. Each well was scored manually using a dissecting microscope. To minimize the influence of plate-to-plate variation of the scoring results, two negative control wells (E3 + 1% DMSO and 1% Pen/Strep) and two positive control wells (20 μM DEAB) were included in each 24-well plate. For each well, the number of mutant embryos was recorded, along with the level of *gata1* expression relative to the positive and negative controls for that plate. If the expression was deemed to be increased in comparison to the negative control wells, the level of increase was scored based on the intensity of *gata1* staining in the mutant embryos and the percentage of total mutants with increased *gata1*. We assigned each compound to a distinct level of rescue (Fig. 2). Level 3 compounds had the strongest *gata1* expression with >50% of mutant embryos uniformly displaying as much or more *gata1* expression as the positive controls from the same plate. Level 2 compounds had >50% of mutant embryos with increased *gata1* compared with negative controls, but the level of *gata1* expression was mixed, with some embryos displaying a high level of *gata1* expression and others less. Finally, level 1 compounds had <50% of mutant embryos with increased *gata1* and a generally weakly increased degree of *gata1* expression when compared with negative controls (Fig. 2).

Whole-mount *in situ* hybridization. Whole mount *in situ* hybridizations were performed as previously described using

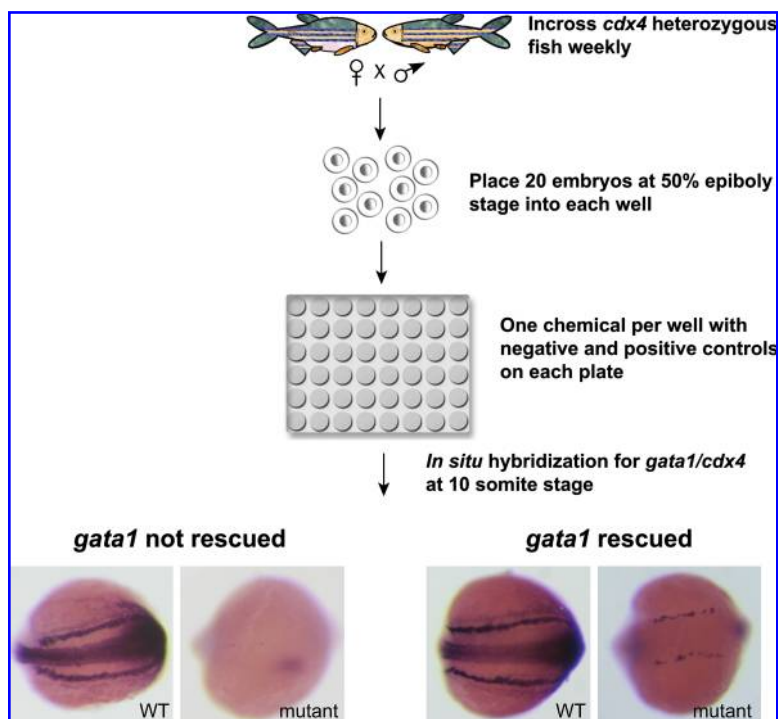


FIG. 1. Screen design. *cdx4* heterozygous adult fish were incrossed weekly to generate embryos for the chemical screen. Chemicals from the Institute of Chemistry and Cell Biology library were transferred by robotics from 384-well plates to 48-well plates. Each plate contained negative (1% DMSO) and positive (20 μ M DEAB) control wells. About 20 synchronized embryos at 50% epiboly stage were placed manually into each well of the 48-well plates. Once the embryos reached the 10-somite stage, they were fixed and whole-mount *in situ* hybridization was performed with both *gata1* and *cdx4* riboprobes. Each well was examined and scored manually. A posterior view of representative embryos is shown, with anterior to the left and posterior to the right. *cdx4*-mutant embryos were identified by lack of *cdx4* expression. In most wells, *cdx4*-mutant embryos had little or no *gata1* expression. The wells with increased *gata1* expression in the *cdx4*-mutant embryos were scored as a "rescue." DMSO, dimethyl sulfoxide; DEAB, 4-diethylamino benzaldehyde; WT, wild type. Color images available online at www.liebertonline.com/zeb.

antisense riboprobes labeled with digoxigenin and detected with antidigoxigenin antibody conjugated to alkaline phosphatase.²² The following antisense riboprobes were generated as described: *cdx4*, *hoxa9a*, *hoxb7a*,¹⁸ *fli1*,²³ *gata1*,¹⁷ *krox-20*,²⁴ *myoD*,²⁵ *pu.1*,¹⁶ and *scl*.²⁶ The *in situ* protocol was performed using the Biolane™ HTI machine (Holle & Huttner AG).

Genotyping. Genomic DNA was isolated from fin clips or whole embryos as previously described.²⁷ *cdx4* heterozygous fish and homozygous mutant embryos were genotyped as previously described.¹⁸

Results

Chemical screen to identify *cdx4* modifier phenotype

A total of 2640 compounds from the known bioactive collection of the ICCB were tested. This same panel of compounds had been tested previously in our laboratory during a screen to identify small molecules that alter *runx1* and *c-myb* expression during definitive hematopoiesis.^{5,10} Each compound was tested individually in a single well, because pre-

liminary experiments with pooled compounds yielded high levels of toxicity (data not shown). When tested individually in our assay, 42 compounds were noted to cause developmental delay (1.6%), 24 were teratogenic (0.9%), and 72 were toxic to embryos (2.7%), resulting in death of all or most embryos in the well.

A total of 5 compounds were scored as level 3 hits, 16 compounds as level 2 hits, and 18 compounds as level 1 hits. All the level 2 and level 3 compounds and most of the level 1 compounds were purchased and a range of concentrations were retested in the *gata1/cdx4* *in situ* hybridization assay. Two of the level 3 compounds, bergapten and asarylaldehyde, had reproducible rescue of *gata1* expression in *cdx4* mutants, whereas none of the results with level 1 or 2 compounds could be repeated convincingly.

A small percentage (0.9%) of chemicals tested had only wild-type embryos in the well, and thus no *cdx4* mutants to score. On the unlikely possibility that the chemical exposure had completely rescued the *cdx4*-mutant phenotype rendering the *cdx4*-mutant animal indistinguishable from the wild-type, the embryos in these otherwise unscorable wells were

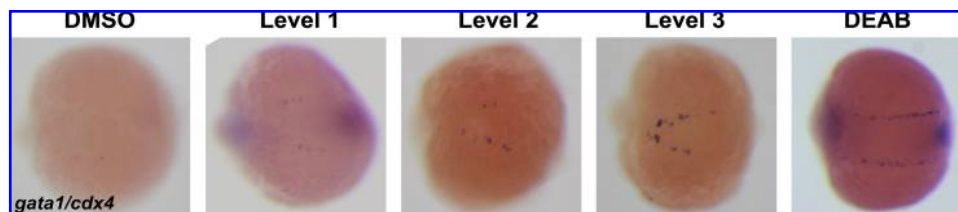
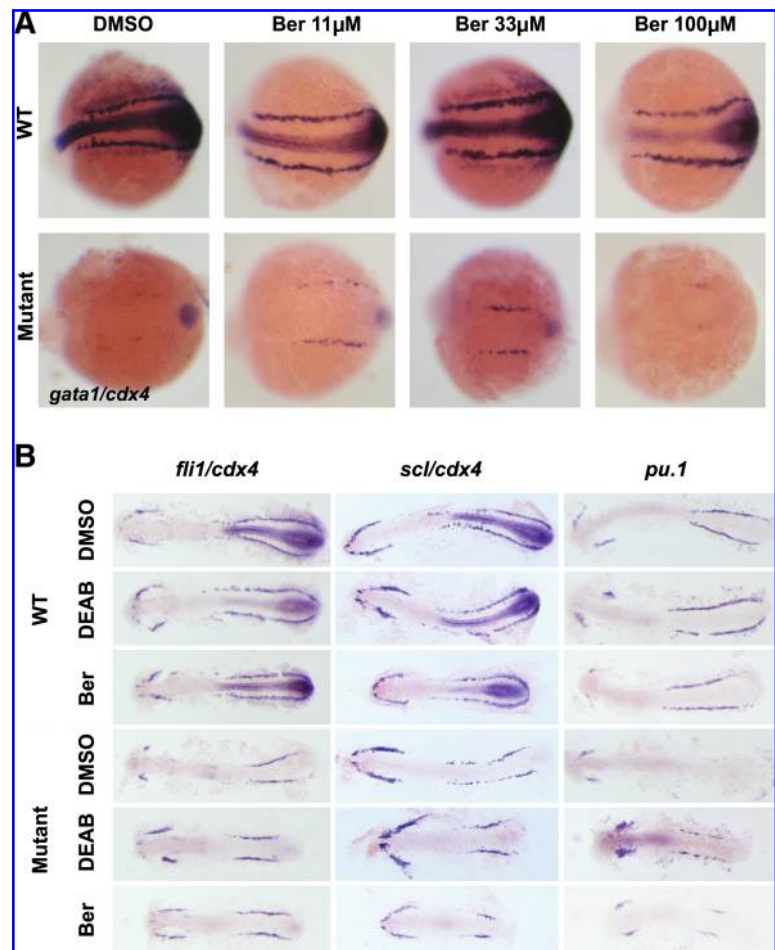


FIG. 2. Scoring system for rescued embryos. The level of *gata1* rescue was determined by the percentage of rescued mutant embryos in each well and by the intensity of *gata1* expression in these embryos in comparison to the negative and positive control wells for each individual plate. DMSO vehicle was the negative control, and 20 μ M DEAB was the positive control. Representative mutant embryos are shown after *in situ* hybridization for *gata1* and *cdx4* expression. Color images available online at www.liebertonline.com/zeb.

FIG. 3. Bergapten increases *gata1* and *pu.1* in *cdx4*-mutant embryos. All embryos are displayed with anterior to the left and posterior to the right. (A) *cdx4* heterozygous adults were incrossed, and the resulting embryos were treated with varying doses of bergapten. Bergapten rescues *gata1* expression (level 3) in the *cdx4*-mutant embryos, and this rescue is most effective at 33 μ M. Whole-mount embryos at the 10-somite stage are displayed from a representative experiment. (B) *cdx4* heterozygous adults were incrossed, and the resulting embryos were treated with DMSO vehicle control, 20 μ M DEAB, or 30 μ M bergapten. Flatmounted embryos at the 10-somite stage are shown. WT embryos and *cdx4* mutants stained with *pu.1* were confirmed by genotyping. Bergapten does not change the expression pattern of mesodermal genes *fli1*, *scl*, and *pu.1* in WT embryos. In *cdx4*-mutant embryos, however, *pu.1* expression is rescued in the posterior lateral mesoderm when either DEAB or bergapten is applied. Ber, bergapten. Color images available online at www.liebertonline.com/zeb.



genotyped to confirm they were all wild type or heterozygous for the mutant *cdx4* allele. In all cases no homozygous mutant embryos with a wild-type phenotype were identified. Each of these compounds were then retested, and none had *cdx4*-mutant embryos with increased *gata1* expression on subsequent testing.

Bergapten rescues *gata1* expression in *cdx4*-mutant embryos but has minimal effect on the expression of other posterior mesodermal genes

Bergapten and asarylaldehyde were found to increase *gata1* expression in *cdx4*-mutant embryos. We decided to focus on bergapten only, because asarylaldehyde had a very simple chemical structure, such that we could not identify related compounds to study. Bergapten, also known as 5-MOP, is a member of the psoralen family, which is found in bergamont oil.²⁸ Psoralen family members have been utilized in the treatment for psoriasis and cutaneous T-cell lymphoma, in combination with ultraviolet A (UVA) phototherapy (reviewed in Ref.²⁹). The original concentration of bergapten in the screening well was estimated to have been approximately 30 μ M. When a serial dilution of purchased bergapten was tested, maximal rescue of *gata1* expression was observed at 33 μ M, whereas treatment with 100 μ M bergapten was toxic (Fig. 3A). In one representative experiment, 36% of the embryos treated with 100 μ M bergapten were dead by the 10-

somite stage (24/67), and 51% of the live embryos were dysmorphic (22/43).

To determine whether bergapten also affected other posterior tissues in the developing embryo, we examined the expression patterns of multiple mesodermal genes, including *fli1*, *scl*, and *pu.1*, in *cdx4*-mutant embryos treated with bergapten (Fig. 3B). In wild-type embryos, all of these genes are expressed in the ALM and PLM from the three-somite stage.^{23,26} *fli1* marks vascular progenitors, whereas *scl* is expressed by both vascular and hematopoietic cells, and *pu.1* is expressed by committed myeloid cells.^{15–16} The *cdx4*-mutant embryos have decreased expression of *fli1* and *scl* in the PLM, and almost normal expression in the ALM. When treated with either DEAB or bergapten, there was no observable difference between the expression level of *fli1* or *scl* in untreated wild-type embryos and embryos treated with DEAB or bergapten. Although the overall expression domain in the posterior mesoderm is shorter in the *cdx4*-mutant embryos treated with DEAB or bergapten, the intensity of expression is unchanged compared with untreated *cdx4* mutants (Fig. 3B). In contrast, the expression of *pu.1* is absent in the PLM of *cdx4* mutants and appears relatively normal in the ALM. When treated with bergapten, *cdx4*-mutant embryos have increased expression of *pu.1* in the PLM, similar to the rescue effect observed with *gata1* expression. This same result is also evident after incubation with DEAB. This indicates that both DEAB and bergapten may expand primitive blood development by acting

upon a common erythromyeloid progenitor, but these compounds do not appear to act upon hemangioblasts.

Bergapten disrupts A-P patterning

The morphology of the *gata1*+ stripes in the posterior mesoderm was similar in both DEAB-treated and bergapten-treated embryos, but the anterior portion did not merge toward the midline as seen in wild-type embryos at the 10-somite stage. We hypothesized that bergapten and DEAB would have similar effects on A-P patterning overall. Having observed that posterior expression of *gata1*, *pu.1*, *scl*, and *flil* had similar patterns for embryos treated with either DEAB or bergapten, we examined anterior patterning more closely by studying the expression of *krox-20* and *myoD*. As *krox-20* stains rhombomeres 3 and 5 in the hindbrain, whereas *myoD* stains the somites, this allowed a measurement of the distance between the hindbrain and the most anterior somite pair. Previous studies have shown this distance between *krox-20* and *myoD* to be highly sensitive to RA concentration, as demonstrated in mutants with disrupted RA signaling.³⁰ Likewise, treatment of wild-type embryos with DEAB is reported to have a similar effect.⁷ When treated with DEAB, the gap between *krox-20* and *myoD* expression disappears, indicating defects in the posterior hindbrain. This result is identical for embryos treated with 33 μ M bergapten, whereas a clear gap is noted in the untreated control embryos (138.6 \pm 7.6 μ m) (Fig. 4B). These data indicate that bergapten and DEAB have similar effects on A-P patterning. When the same *krox-20*–*myoD* assay was conducted with embryos obtained from an incross of *cdx4* heterozygous animals, embryos were found to lose the gap between *krox-20* and *myoD* re-

gardless of their genotype. However, in the *cdx4*-mutant embryos, *krox-20* expression overlapped with that of somite 1 and 2, indicating that A-P patterning in the *cdx4*-mutant embryos was more severely affected compared with their siblings (Supplemental Fig. S1, available online at www.liebertonline.com). As DEAB is known to inhibit RA biosynthesis,^{19,31} it is possible that bergapten may also inhibit the RA pathway through an unknown mechanism.

8-MOP has similar effect as bergapten on *gata1* expression, whereas PAP-1 does not

To determine if structurally similar psoralen compounds related to bergapten may have similar effects on hematopoiesis, 8-MOP and PAP-1 were tested in these same assays. Like bergapten, 8-MOP is used clinically to treat psoriasis and has DNA intercalating activity (reviewed in Ref.²⁹). In contrast, PAP-1 is a potassium channel blocker, which is also used for clinical treatment of psoriasis, but with decreased phototoxicity due to bulky side groups attached to the psora ring³² (Fig. 4A).

We first tested if 8-MOP and PAP-1 also affect A-P patterning in wild-type embryos using the *krox-20*–*myoD* assay mentioned earlier. As expected, 8-MOP disrupts the posterior hindbrain for all doses tested, with maximal effect at 33 μ M, albeit at lesser degree when compared with bergapten-treated embryos (average *krox-20*/*myoD* distance of 8-MOP-treated embryos = 85.8 \pm 4 μ m) (Fig. 4B; data not shown). In contrast, PAP-1 does not affect the distance between *krox-20* and *myoD* (average distance = 141.2 \pm 13 μ m). When we examined the ability of 8-MOP and PAP-1 to rescue *gata1* expression in *cdx4*-mutant embryos, 8-MOP increased *gata1* expression at 33 μ M, whereas PAP-1 did not (Fig. 4C). Overall, 8-MOP seems to act

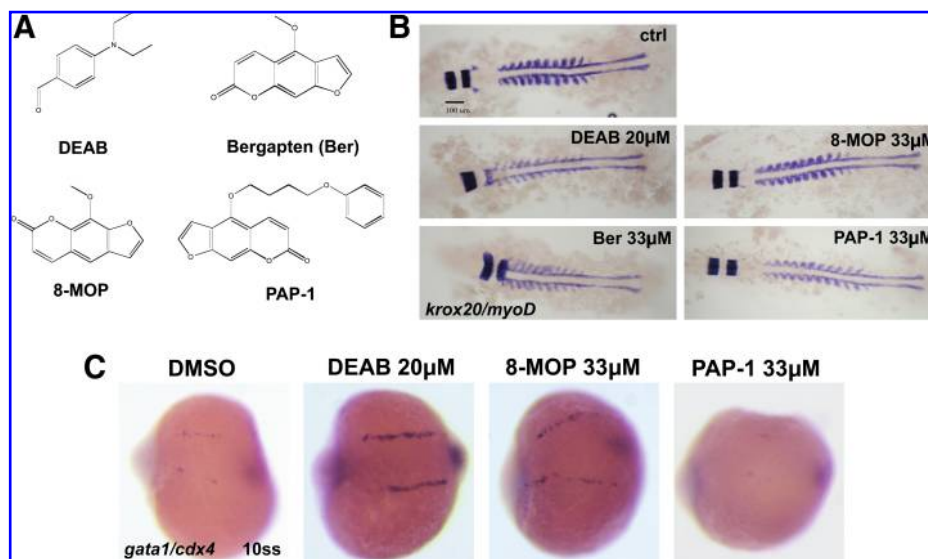


FIG. 4. Bergapten and other psoralen compounds affect anterior–posterior patterning. (A) Chemical structures of the compounds tested. (B) Whole-mount *in situ* hybridization of WT AB embryos with *krox-20* and *myoD* riboprobes. Whole-mount 10-somite stage embryos are shown from a representative experiment. The average distance between *krox-20* and *myoD* in control embryos is 138.6 μ m (\pm 7.6 μ m). The bergapten-treated embryos lose the gap between *krox-20* and *myoD*, similar to DEAB-treated embryos. 8-MOP also affects the distance, but to a lesser degree (85.8 \pm 4 μ m). In contrast, PAP-1 does not affect this distance. (C) *cdx4* heterozygous adults were incrossed, and the resulting embryos were treated with DMSO vehicle control, 20 μ M DEAB, 33 μ M 8-MOP, or 33 μ M PAP-1. Whole-mount *in situ* hybridization was done with *gata1* and *cdx4* riboprobes at the 10-somite stage. 8-MOP robustly rescues *gata1* expression in the *cdx4*-mutant embryos, whereas PAP-1 does not rescue. ss, somite stage; PAP-1, 5-(4-phenoxybutoxy)psoralen; 8-MOP, 8-methoxypsoralen. Color images available online at www.liebertonline.com/zeb.

similarly to bergapten, as the effect of 8-MOP on other mesodermal lineage gene expressions, including *scl*, *fli1*, and *pu.1*, was identical to bergapten (Supplemental Fig. S2, available online at www.liebertonline.com).

Discussion

In this study, we conducted a chemical modifier screen in the *cdx4* mutant background to identify pathways that interact with the *cdx4* pathway in regulating primitive hematopoiesis. On screening a library of known bioactive compounds, bergapten, a member of the psoralen family, was found to increase *gata1* expression in *cdx4*-mutant embryos. Further investigation of additional psoralen family members, including 8-MOP and PAP-1, revealed that 8-MOP acts similarly to increase *gata1*, albeit less robustly than bergapten. In addition to the *gata1* rescue, these compounds also affected A-P patterning, particularly of the hindbrain, as evidenced by the decreased distance between *krox-20* and *myoD* expression pattern in embryos treated with psoralen family members. This pattern was reminiscent of embryos treated with an inhibitor of RA biosynthesis, as well as genetic mutants with disruption in RA signaling.^{30,33}

Inhibitors of RA signaling, such as DEAB, have been shown previously to increase *gata1* expression during primitive hematopoiesis in zebrafish (J.L.O. de Jong and L.I. Zon, unpublished data). Taken together, these results suggest that the mechanism of *gata1* rescue by psoralen compounds may also result from inhibitory effects on the RA pathway. No direct interaction is known between psoralens and retinoids, although both are used to treat diseases of the skin, such as psoriasis. Case reports suggest that there may be an additive or even synergistic effect when using both retinoids and psoralens with UVA to treat scleroderma.³⁴ Another report has shown that psoralens with UVA increase the expression of CYP2S1, a cytochrome P450 enzyme that is predominant for the metabolism of RA in the skin.³⁵ If an analogous mechanism were present in our assay, then bergapten may induce the expression of *cyp26a1* or another metabolizing enzyme in the developing zebrafish, thereby decreasing the effective RA concentration by enhanced biodegradation.

To test if bergapten disrupts RA signaling, an epistasis experiment was conducted by simultaneously treating wild-type embryos with both bergapten and RA. The expression pattern of *krox-20* and *myoD* was the same for embryos treated with RA alone (data not shown), indicating that bergapten does not suppress exogenous RA signaling, and suggesting that if bergapten does affect the RA pathway, it most likely functions upstream of *raldh2*, the rate-limiting enzyme for RA biosynthesis.^{33,36} It could also be possible that while bergapten induces *cyp26a1*, the RA-metabolizing enzyme, the level of *cyp26a1* may not be sufficient to metabolize a high concentration of exogenous RA. Additional experiments are needed to determine whether the activity of the psoralens in blood development is due to inhibition of RA biosynthesis, activation of RA metabolism, or a parallel pathway.

In addition to the RA pathway, we also examined if bergapten rescues *gata1* via the *hox* pathway. It has been previously reported that *cdx4* homozygous mutant embryos lose posterior *hox* gene expression, and that *hoxb7a* and *hoxa9a* overexpression in these embryos rescue *gata1* expression.¹⁸ However, when examined by *in situ* hybridization, the levels

of *hoxb7a* and *hoxa9a* RNA expression in the control and bergapten-treated embryos were identical (Supplemental Fig. S3, available online at www.liebertonline.com). This indicates that bergapten rescues *gata1* expression via a mechanism that is independent of the *hox* pathways.

Multiple steps need to be carefully considered before performing a modifier screen on a mutant background. First is the variability in the rescue phenotype. During this screen, significant plate-to-plate variability of *gata1* expression in the untreated mutant embryos was noted. This was likely due to slight differences in staging and nonsynchronized embryos. Another influencing factor was the *in situ* hybridization step, which depended on the quality of the riboprobe used and the length of staining time. Of note, even with the most robust rescues, increased *gata1* expression was never observed in 100% of the *cdx4*-mutant embryos in a given well. Using 50% as the threshold proved to be adequate to identify legitimate hits.

A second point to be considered is the toxicity of chemicals to the embryos. The overall toxicity detected in our screen was 5.2%. This was more than double the 2% toxicity level experienced by North *et al.* (T.E. North, pers. comm.), who screened the same chemical library we did. Of note, in our screen, embryos were incubated at an earlier stage than in the screen by North *et al.*, whose embryos were incubated from the three-somite stage to 36 h postfertilization.⁵ Not surprisingly, incubation during gastrulation resulted in a higher rate of toxicity. This should be taken into account when designing a chemical screen, along with the stages when the mutant gene is anticipated to have the most influence. For example, as *cdx4* has important A-P patterning effects during gastrulation that set up the expression levels of the posterior *hox* genes, our screen was designed to include chemical exposure during gastrulation. The effect of bergapten on *gata1* is diminished when added to embryos after completing gastrulation (data not shown), and so if our screen had been designed for later chemical exposure time, we may not have detected bergapten as a hit.

Chemical modifier screens using a mutant background can provide useful knowledge about the affected pathways in that mutant, particularly related to a specific phenotype of interest. We have shown in our study that the loss of *gata1* and *pu.1* expression in the *cdx4*-mutant embryos is rescued by changing A-P axis development. The zebrafish community has many mutants with hematopoietic defects that are affected at various steps during hematopoietic development, including mutants with defective hematopoietic stem cells and mutants with problems at later stages of blood differentiation. Conducting a chemical modifier screen in these various mutants will increase our understanding of normal hematopoietic development and may provide insight into aberrant development and hematopoietic malignancies.

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Disclosure Statement

L.I.Z. is a founder and stockholder of Fate, Inc. and a scientific advisor for Stemgent.

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